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DISTRIBUTION AND INTERRELATIONS OF PERIPHERAL ADRENERGIC AND NON-ADRENERGIC NERVE TERMINALS

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The present summary is based on the following papers:

1) EHINGER, B., and SPORRONG, B. (1966) Adrenergic and cholinergic axons in the mouse iris. Experientia 22, 218.

2) EHINGER, B., FALCK, B., and SPORRONG, B. (1966) Adrenergic fibres to the heart and to peripheral vessels. Bibl. anat. 8, 35--45.

3) EHINGER, B., SPORRONG, B., and STENEVI, U. (1968) Simultaneous demonstration of adrenergic and non-adrenergic nerve fibres. Histochemie 13, 105-110.

4) EHINGER, B., FALCK, B., PERSSON, H., and SPORRONG, B. (1968) Adrenergic and cholinesterase-containing neurons in the heart. Histochemie 16, 197-205.

5) EHINGER, B., FALCK, B., PERSSON, H., ROSENGREN, E., and SPORPONG, B. (1970) Localization of acetylcholine in the feline iris. J. Physiol. (In press).

6) EHINGER, B., FALCK, B., and SPORRONG, B. (1970) Possible axoaxonal synapses between peripheral adrenergic and cholinergic nerve terminals. Z.Zellforsch. 107, 508-521.

7) NIELSEN, K.C., OWMAN, CH., and SPORRONG, B. Ultrastructure of the autonomic innervation apparatus in the main pial arteries of rats and cats (In press).

8) NILSSON, E., and SPORRONG, B. Electron microscopic investigation of adrenergic and cholinergic axons in the rabbit SA--node (In press).

9) NIELSEN, K.C., OWMAN, CH., and SPORRONG, B. Sympathetic nervous control of pial arteries: Tyramine-induced contraction of the isolated middle cerebral artery of the cat (In press).

These papers will be referred to by their Arabian numerals.

Abbreviations used:

ACh = acetylcholine

AChE = acetylcholinesterase (specific cholinesterase)

ChE = cholinesterase (non-specific cholinesterase)

HC-3 = hemicholinium 3

DA = dopamine

NA = noradrenaline

SA-node= sino-atrial node

AV-node= atrio-ventricular node

INTRODUCTION

In 1959, Burn and Rand advanced an interesting hypothesis concerning the release of ACh and NA from nerve terminals in the peripheral autonomic nervous system. This hypothesis suggests that a postganglionic sympathetic cholinergic nerve, upon electrical stimulation or stimulation by ACh-releasing drugs, releases ACh, which in turn releases NA from a pool in or around the sympathetic nerve terminal (Burn and Rand 1959, 1965).

The theory was evolved to explain certain pharmacological results that could not be explained by the ordinary neurotransmission theory. Thus Hoffmann, Middleton and Talensik as early as 1945 found that ACh given to the atropinized isolated heart caused a positive ino- and chronotropic effect that was accompanied by the appearance of an adrenaline-like substance in the perfusate leaving the heart. In 1953, Kottegoda, using nicotine instead of ACh, observed the same on the isolated atropinized rabbit atria. He also showed that this action of nicotine could be blocked by hexamethonium. This led to the idea that nicotine-like agents excited intrinsic adrenergic ganglia in the heart, postulated by Dixon (1924); ~~and Loadors and Long (1962)~~; Later Burn and Rand (1958) showed that this nicotine effect depended on an intact amount of NA in the organ used, since the sympathomimetic effect of nicotine was totally lacking if the animal had been pretreated with reserpine, or if the organ had been previoslydenervated sympathetically (Lee and Shideman 1959 a and b). Later pharmacological evidence for this theory was obtained from a great number of organs and species such as ileum, colon, blood vessels, nictitating membrane, bronchi, piloerectors, and spleen (for ref. see Burn and Rand 1965, Ferry 1966, and Burn 1968).

However, the interpretation of all pharmacological results did not support the theory. Thus Leaders and Long (1962) investigated the positive chronotropic effect of nicotine in non-atropinized cat and rabbit atria-nerve preparations. They showed that HC-3 abolished both the negative and the positive chronotropic effect of nicotine and postulated the involvement of the parasympathetic nervous system for the response. Later, Leaders (1963) and Leaders and Dayrit (1965) suggested another explanation of the pharmacological inconsistencies earlier described. They suggested the possibility of a local interaction mechanism between adrenergic and cholinergic terminals where the two different nerve types can exert a mutual influence.

In support of the Burn and Rand hypothesis, it has been emphasized that anticholinesterase agents can potentiate the effect of sympathetic stimulation (Burn and Rand 1965; Burn 1968). However, Thoenen, Tranzer, Hürlimann and Haefely (1966) demonstrated that, in the purely adrenergically innervated cat spleen capsule, anticholinesterase agents could not potentiate the effect of sympathetic stimulation as they do in many other organs with a double, cholinergic and adrenergic nerve supply.

The pharmacological results in favour of or opposed to the cholinergic link theory have been very contradictory, as reviewed by Ferry (1966). On purely pharmacological evidence, it is at present impossible to accept or deny the existence of a cholinergic link in adrenergic nerves in general. It is equally difficult to say whether the theory is true for some part of the body or some species, since the individual evidence for or against the theory has been gathered from a vast number of species and organs, some of which even containing both pre- and postganglionic fibres. Moreover, it has not yet been possible to establish chemically the existence of either the synthesizing enzyme, choline acetylase, or the transmitter, acetylcholine, in the adrenergic terminals, the ultimate proofs of the

existence of ACh in adrenergic nerves (Hebb 1961; Ferry 1966).

The interpretation of the morphological findings has not been unanimous. Thus Eränkö and Härkönen (1964), Eränkö and Räisänen (1965), and Jacobowitz and Koelle (1965) studied different species and organs with a modified fluorescence method which permits the demonstration of both catecholamines and AChE in the same section. They all concluded that the participation of ACh in adrenergic transmission was highly probable in many, although not all, organs and species studied. However, they could not exclude the possibility that ACh and NA in different nerves were situated so close together as to permit a mutual interaction, as suggested by Ehinger and Falck 1966, who used a similar method combined with selective denervation on the rat iris. Also, Hamberger _et al_. 1963 were unable to detect histochemically any AChE in adrenergic pericarya.

MATERIAL AND METHODS

Animals: The experimental material consisted of 24 mice, 107 rats, 56 cats, 13 guinea-pigs, 50 rabbits, and 3 monkeys.

Fluorescence microscopy: Tissue pieces were quenched in liquid propanepropylene mixture cooled by liquid nitrogen, freeze-dried, treated with gaseous formaldehyde at 80°C generated from paraformaldehyde for 1 or 3 hours, embedded in paraffin in vacuo, and sectioned serially at 3-10 μ thickness. Before fluorescence microscopy, the preparations were mounted in Entellan (Merck) containing xylene. (For details, see Falck and Owman 1965 or Björklund, Falck and Owman 1970).

Methylene blue: Irides were incubated at 37°C in methylene blue (Merck), Metylenblau für medizinische Zwecke) solutions of various compositions containing NA-bitartrate (1 μg/ml) through which oxygen or carbogen (95% O_2+5% CO_2) were bubbled continuously. Some irides were then stretched on microscope slides, rapidly air-dried and desiccated for one hour at room temperature over phosphorus pentoxide, and treated with gaseous formaldehyde as described under "Fluorescence Microscopy". In another series of experiments, the aerated methylene blue solution was administered intra-arterially with clamping of uninteresting vascular areas. The irides were rapidly dissected out, stretched on microscope slides, air-dried, and examined in the light microscope (Paper 3).

Acetylcholinesterase techniques

Whole mounts from heart valves were dried and treated with formaldehyde gas and stained for cholinesterase according to Ehinger and Falck (1966) for the demonstration of adrenergic and acetylcholinesterase-containing nerve fibres in the same specimen. Cryostat sections were stained for cholinesterases according to Holmstedt (1957). As inhibitors, were used: iso-OMPA (tetra-isopropylpyrophosphoramide, 10^{-5}M), mipafox (N,N-di-isopropylphosphoramide fluoride 4 x 10^{-6}M), and NU-683 (dimethylcarbamate of (2-hyd-

roxy-5-phenylbenzyl)-trimethylammoniumbromide = Ro 2-0683, Hoffmann-La Roche, 3×10^{-8}M).

Denervation techniques

For parasympathetic denervation of irides, the ciliary ganglion was removed unilaterally 4-12 days before killing (Ehinger and Falck 1966). In the rat, the success of the operation was checked by identifying the ciliary ganglion in the excised tissue with routine histological methods.

Sympathectomy was performed by the unilateral excision of the superior cervical ganglion and as much as possible of the sympathetic chain cranial to the ganglion.

Electron microscopy

Animals were injected with 5-hydroxydopamine, 100 mg/kg being injected intraperitoneally 4 hours before killing or daily for up to four days with the last injection 4 hours before sacrifice. Tissue pieces were fixed in 2 per cent glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for one hour, postfixed in 2 per cent osmium tetroxide in the same buffer, and embedded in araldite (Durcopan ACM; Fluka AG) or in Vestopal W. Tissues from non-injected animals were fixed in 2 per cent $KMnO_4$ in veronal buffer according to Luft and embedded in Vestopal W. The tissues were contrasted either en bloc with uranyl acetate and phosphotungstic acid before embedding or with lead citrate in the sections. Specimens thus treated achieve certain morphological characteristics which make it possible to differentiate between adrenergic and non-adrenergic nerve terminals. In $KMnO_4$-fixed tissues, the adrenergic terminal swellings, the so called varicosities, are filled with numerous small vesicles, almost all containing an electron-dense core (Richardson, 1966; Hökfelt, 1967; Hökfelt and Jonsson 1968). These so-called synaptic vesicles are of two kinds, one large, about 1000 Å in diameter, and one small, about 400-500 Å in diameter. The 400-500 Å type is seen within the

varicosities where they are very numerous. The 1000 Å large type is seen in preterminal axons as well as in the varicosities and in the intervaricose parts of the terminal, usually only one or rarely two to three being seen in each axon cross section.

After the treatment of animals with 5-hydroxydopamine followed by glutaraldehyde and osmium fixation, dense-cored vesicles are seen in the adrenergic terminals as after $KMnO_4$-fixation. The main part of the small vesicles are about 400-500 Å in size, although larger ones up to 800 Å are sometimes seen. However, with this treatment, not all the vesicles of this size achieve a dense core; many of them appear empty. ~~Another type of dense core; many of them appear empty~~ Another type of dense cored vesicle is also seen, 1000 Å in size, with approximately the same appearance and frequency as in $KMnO_4$-fixed tissues. However, this type of granules is not specific for adrenergic nerves in tissues fixed in glutaraldehyde and osmium (Bloom and Aghajanjan 1968; Hökfelt 1969).

Determination of acetylcholine

The acetylcholine was extracted in 2 ml trichloroacetic acid and washed with ether. The residual ether was removed by vacuum evaporation. The volumes of the extracts were adjusted to 2 ml with distilled water. The extracts were then assayed for acetylcholine on the eserinized dorsal muscle preparation of the leech in a 15 ml bath.

Physiological localization of the SA-node

The sino-atrial node was localized and outlined electrophysiologically by the insertion of a microelectrode in and around the node according to Trautwein and Uchizono (1963). The part giving typical potentials was dissected out, fixed in $KMnO_4$, and processed for electronmicroscopy as described above.

Effect of drugs on middle cerebral artery

The method used is described in detail by Nielsen and Owman (1970). A portion of the proximal part of the middle

cerebral artery of cats was mounted in a 15 ml Krebs-Ringer bath kept at 37.5 $\pm$ 0.5^{o}C pH = 7.30-7.40. The arrangement permitted registration of circular contractions in the vessel.

Before testing, the artery was placed under a tension of 300-400 dyn, which proved adequate to reveal both constriction and relaxation; it was allowed to stabilize for 30-60 min. Solutions of the drugs to be tested were added to the bath in amounts of 0.1--1.0 ml.

RESULTS

Heart (papers 2,4,6,8)

The hearts of all the studied species receive a rich adrenergic as well as cholinergic nerve supply, which varies somewhat between the different parts of the heart. In general, the atrial myocardium contains more adrenergic terminals than the ventricular myocardium. The epicardium on the posterior surface of the atria and upper ventricles shows many smooth adrenergic preterminals with a faint green fluorescence. These bundles often contain some beaded, strongly fluorescent terminals. Non-fluorescent ganglion cells are frequently seen in this region and also scattered among the muscle cells of the atria and ventricles, especially in the posterior parts adjacent to the anulus fibrosus. These ganglion cells are often surrounded by a cocoon of delicate adrenergic terminals, as described elsewhere (Jacobowitz 1967, Norberg 1967). In spite of deliberate search, no fluorescent adrenergic ganglion cells were ever found in the heart or in its immediate surroundings. The existence of a considerable adrenergic innervation through the vagus nerve was excluded by crushing the vagus in rabbits, showing only comparatively few adrenergic nerves, mainly localized around vessels (paper 4).

The general distribution of cholinergic (i.e. cholinesterase containing) nerves follows the patterns of adrenergic terminals, the atria containing more than the ventricles.

In all studied species, small intensely fluorescent cells were seen in and around the atria. They were often seen in the ganglia but also along nerve trunks in the adipose tissue around the heart, in the pericardial connective tissue, and even among the atrial muscle cells. Irrespective of their location, they always had the same morphological appearance, often with short, stout processes. Microspectrographically these cells were shown to contain a primary

catecholamine, most probably dopamine. These cells are undoubtedly of the same type as described by Jacobowitz (1967).

The SA and AV-node, as well as the bundle of His, receive an intense adrenergic and cholinergic nerve supply, as seen by the light microscopical (paper 2,4) and electron microscopical (paper 8) investigations. Light microscopically, they are characterized by the intense adrenergic and cholinergic innervation patterns, easily discernible from the lesser amount of nerves found in the surrounding myocardium, each muscle cell in these specialized locations apparently receiving at least one and probably several terminals of both kinds. These innervation patterns seen with the light microscope were common to all studied species.

Electron microscopy revealed many great nerve bundles containing myelinated and unmyelinated preterminals (i.e. lacking varicosities and synaptic vesicles) in the collagenous surroundings of the SA node, each terminal being isolated from the others by the Schwann cell. Scattered among these preterminals, varicosities are frequently seen, containing an abundance of 400-500 Å synaptic vesicles, either dense-cored or empty. ~~Close to the pacemaker region are a great many small nerve bundles consisting almost exclusively of varicosities containing synaptic vesicles predominantly of either dense-cored or empty~~ Close to the pacemaker region are a great many small nerve bundles consisting almost exclusively of varicosities containing synaptic vesicles predominantly of either dense-cored or empty type mixed together in the same bundle. Here, the Schwann cell is split up or lacking between the individual nerve terminals, allowing varicosities of similar or different kind to come in close apposition to each other, the distance between adjacent membranes being in the order of 200-250 Å. Often, a moderately electron-dense material is found in this narrow cleft. The membrane specializations characterizing a synapse in the C.N.S. or in ganglia, however, have not been observed. A slight thickening of that part of a membrane facing another varicosity can sometimes

be seen, but it does not appear in every section.

For long distances, the pacemaker cells lie very close to each other, the distance between adjacent membranes being about 200 Å. There is often a widening of this narrow cleft, making impressions in the surrounding pacemaker cells. This expanded region is always occupied by an adrenergic or a cholinergic axon lacking Schwann cell envelope and only surrounded by its basement membrane. The basement membrane of the terminal seems partially to fuse with the basement membranes of the surrounding pacemaker cells, and gives rise to a moderately electron-dense material between the terminal and the pacemaker cells. The distance between adjacent membranes of the varicosity and its surrounding cells is again in the order of 200--250 Å. Greater impressions that form small invaginations in the pacemaker cells are frequently seen on those sides that are not in apposition to other cells. These furrows contain one to several nerve terminals of both kinds, which come in close apposition to each other and to the muscle cell. Here too, the Schwann cell envelope is incomplete or totally lacking.

The rest of the heart contains a distinctly less well developed innervation, both adrenergic and cholinergic, as seen with the light microscope. Subendocardially, the heart shows a single--layered nerve plexus containing both adrenergic and cholinergic nerve terminals. On the atrial side of the atrioventricular valves, a corresponding well-developed adrenergic and cholinergic nerve plexus is found with numerous connexions to the atrial subendocardial plexus. A similar adrenergic, as well as cholinergic, nerve plexus is also seen in the semilunar valves, but not to the same extent, and with individual variations between different species. The combined demonstration of AChE and fluorescence on the same stretch preparation of semilunar and atrioventricular valves shows that the two nerve nets are usually, although not always, congruent.

The myocardium proper receives a fair amount of both

adrenergic and cholinergic nerve terminals, the atria more than the ventricles. Injections with indian ink eliminated the possibility of an exclusively vascular innervation.

The existence of adrenergic and cholinergic nerves and their mutual relation was further elucidated by electron microscopy (paper 6). At the base of the atrioventricular valves, many unmyelinated nerves are seen, together with some varicose terminals. Further distally towards the free margins, the terminals predominate with some single unmyelinated nerve trunks. The connective tissue between the heart muscle cells and the smooth muscle cells of the endocardium contains small nerve trunks consisting mainly of varicose terminals. In the parts of the heart close to the anulus fibrosus, these small trunks are often seen together with both myelinated and unmyelinated preterminals. The individual relation between adrenergic and cholinergic terminals is principally of the same type as seen in the SA-node. Towards the terminals part of the axon, the Schwann cell is split up allowing varicosities of the same or different kind to come in close apposition to each other, the distance between two adjacent membranes being in the order of 200-250Å. The presence of single naked axons infiltrating between closely adjacent myocardial cells is rare in the atrial as well as in the ventricular myocardium. Instead, the terminals are grouped together in small bundles containing three to about six terminals of both kinds. These small bundles form hoods upon the margins of the muscle cells facing a collagen-rich interspace between groups of myocardial cells, the distance between the terminals and the muscle cell membrane again being in the order of 200-250 Å. The nerve bundles could also be seen passing along muscle cells, occasionally coming into close apposition to the muscle cell and the accompanying nerve terminals in the same bundle.

Iris (paper 1,3,5,6)

From earlier works, it is well known that the iris of most species contains a rich network of adrenergic and cholinergic

nerve terminals (Falck, 1962; Malmfors, 1965; Ehinger and Falck, 1965, 1966; Laties and Jacobowitz, 1964, 1966; Ehinger, 1967) and that the meshes in the mouse iris almost always contain only one adrenergic nerve fibre (Malmfors 1965). The use of the methylene blue technique according to Hillarp (1946) makes it possible to show all terminals in the meshes (paper 1). It was found that every strand of the ground plexus contained at least two varicose axons, suggesting that the single adrenergic axon is accompanied by one or more non-adrenergic nerve terminals.

Slight modifications of the original methylene blue method make it possible to combine it with the fluorescence method of Falck and Hillarp showing adrenergic and non-adrenergic nerve fibres simultaneously (paper 3). Thus two types of varicose terminals show up. One is fluorescent, forming the well known adrenergic ground plexus. This type does not contain any visible methylene blue. The other type is the non-fluorescent terminal, stained by methylene blue. Both types are visible when there is no noradrenaline in the incubation solution, but the preparations then tend to display one type or the other, showing patches with fluorescent fibres mixed with patches containing only methylene blue stained fibres. This variability is decreased by using noradrenaline in the bath. Also the adrenergic fibres achieve a more intense fluorescence, which results in a fairly strong fluorescence in their intervaricose parts thus making the varicosities less prominent. A faint and diffuse orange background fluorescence that increases upon illumination is also seen, but it does not interfere with the demonstration of nerve fibres. It is less prominent in the freeze-dried and paraffin-embedded specimens.

The adrenergic and methylene blue stained nerve fibres usually, although not always, follow each other closely and give rise to almost identical nerve plexuses. The autonomous ground plexus is thus found to consist of tightly intertwined adrenergic and non--adrenergic terminals, probably situated within the same Schwann cell sheath. However, adrenergic as well as non-adrenergic terminals could sometimes be seen alone. Usually, the varicosities of adjacent adrenergic and non-adrenergic nerves lie staggered, an adrenergic vari-

cosity coinciding with an intervaricose part of the non-adrenergic terminal and vice versa. Of special interest (see discussion) is the innervation pattern of the iridic vessels. These vessels are surrounded by a sheath of adrenergic terminals that form the usual adrenergic ground plexus. Methylene blue stained non-adrenergic fibres are completely lacking. The methylene blue stained fibres that do occur close to the vessels are all situated in the focal plane of the dilator plexus and in direct connexion with its fibres. It is thus highly probable that they belong to the dilator plexus rather than to the vessel.

After parasympathetic denervation, only very few methylene blue stained fibres are seen, whereas the adrenergic nerve plexus seems to be unaltered. Appropriate controls were achieved by incubating the irides from the unoperated side together with the denervated one.

These light microscopical findings were further elucidated by electron microscopy performed on the rat iris (paper 6). Essentially the same relations between adrenergic and non-adrenergic axons were found as in the heart (cf. above). Thus the iridic stroma contains many axon bundles, consisting mainly of preterminals (containing microtubules, few mitochondria, and no synaptic vesicles). Frequently, however, also axon varicosities (containing numerous synaptic vesicles and mitochondria) are seen mixed with the preterminals. The varicosities are sometimes seen coming in close apposition to each other within a Schwann cell sheath. The distance between adjoining membranes in such appositions is 200-250 Å. Closer to the dilator, many small nerve bundles are seen which contain only a few nerve fibres mainly consisting of varicosities. Here, the Schwann cell envelope is split up, allowing the varicosities to come in close apposition to each other, the membrane distance again being in the order of 200-250 Å. As in the heart, the adjoining parts of the membranes are at times slightly thicker and more electron-dense than the rest of the membranes around the varicosities, although the thickenings are not so prominent as in the central nervous system. The membrane specializations were seen

with approximately the same frequency as in the heart (cf. above).

In the rat iris, close appositions between axons or axon varicosities and smooth muscle cells, as described in the rabbit iris (Richardson 1964), were only rarely seen.

After sympathectomy, no 400-500 Å dense-cored vesicles were seen either in tissue pieces fixed in $KMnO_4$ or from animals given 5-hydroxydopamine fixed in glutaraldehyde and osmium. The control side had the same appearance as described above.

Chemical estimate of ACh in iridic adrenergic terminals

The cat iris can be selectively denervated parasympathetically by excising the ciliary ganglion (Ehinger 1967). After such denervation, ACh could no longer be detected in the iris (paper 5). Under the conditions of the experiment this means that parasympathetic denervation gives a reduction to less than 6 per cent of the normal iris content. It thus seems resonable to infer that there is considerably less ACh in adrenergic terminals than in cholinergic ones, less than can be detected with the techniques available at present and at least less than 6 per cent of the content of cholinergic terminals.

Estimations of ACh were also made on sympathetically denervated irides, no significant differences ($P > 0.05$) being found between the operated and the control sides.

Vessels (paper 2,7,9)

Depending on their location within the vascular tree, vessels are innervated by widely different numbers of nerves. With the aid of the fluorescence technique of Falck and Hillarp, certain vascular areas were investigated on their content and distribution of adrenergic nerves (paper 2).

Within the aorta and other elastic arteries, adrenergic fibres are found as a thin sheath in the adventitial tissue just outside the media, and along the vasa vasorum in the outer third of the

media. Since the elastic lamellae are autofluorescent, it is at times somewhat difficult to discern the adrenergic terminals, especially in the higher primates where the autofluorescence is particularly disturbing. However, since the adrenergic terminals are varicose, they, can usually be distinguished from the alastic lamellae, even if their fluorescence is only slightly higher. Moreover, no further adrenergic nerves were revealed after treatment with nialamide and l-dopa or after the borohydride reduction test, which makes it highly improbable that the autofluorescence of the elastic lamellae might mask any considerable number of fluorescent adrenergic terminals.

The muscular arteries are usually surrounded by a compact sheath of adrenergic nerve fibres situated just outside the media, the number of fibres decreasing with decreasing size of the vessel. This diminution is usually gradual, but could be abrupt when a small vessel emerged directly from a larger one with a well-developed sheath of nerves. The smallest arterioles were often accompanied by only one or two fibres; in the latter case, the terminals often ran along opposite sides of the vessel.

In order to visualize the capillary bed, indian ink was injected intravascularly. In certain vascular areas, such as in skeletal muscle, sublingual gland, and connective tissue, it was immediately evident that the capillary bed did not regularly receive an adrenergic supply. Adrenergic nerves were regularly seen along vessels down to the smallest arteriole. Sometimes, however, also smaller vessels were found to be accompanied by an adrenergic varicose fibre. The number of smooth muscle cells in their wall is low, as revealed by conventional staining as well as by phase contrast microscopy. Serial sections always revealed that these vessels were connected to the capillary bed, which lacks adrenergic terminals. The exact nature of these vessels is not known, although they could be postcapillary veins.

Generally, the veins have an adrenergic supply less dense than the corresponding arteries. Veins in skeletal muscles have only

few adrenergic fibres, and the renal and caval veins even less, although whole mounts of the latter vein disclosed a definite adrenergic ground plexus. In some thick-walled, superficial, veins, such as the saphenous, facial, and penile veins, adrenergic nerves are more numerous, lying scattered all through the media, mainly in the strands of connective tissue which are abundant in these veins.

For the electron microscopical studies (paper 7), the main pial arteries of rats and cats were chosen for their intense adrenergic as well as cholinergic innervation as seen by light microscopy (Nielsen and Owman 1967; Lavrentieva, Mchedlishvili and Plechkova 1968). The nerve supply to these vessels has also been studied with electron microscopy (Hagen and Wittkowski 1969).

In the so-called periadventitial tissue, well away from the muscular media of the vessel, large nerve trunks are seen, mainly comprised of unmyelinated as well as myelinated preterminal axons, all surrounded by a Schwann cell sheath (paper 7). Closer to the vessel, nerve bundles are seen, containing both preterminals and terminals of both kinds, as described above. The Schwann cell envelope is here partly split up around the individual axons, allowing axons of the same or different kind to come in close apposition to each other or to preterminals, the distance between adjoining membranes being in the order of 200-250 Å. In the adventitia, just outside the muscular media, small nerve bundles are seen, consisting of varicose terminals together with some single preterminal axons. ~~These small groups are either surrounded together with some single preterminal axons.~~ These small groups are either surrounded by an incomplete Schwann cell sheath, or the sheath is completely lacking, the individual nerve fibres being surrounded only by their basement membrane. The smallest distance observed between the free margins of the varicosities and the muscle cells is in the order of 1000 Å. These bundles are often situated within furrows established by adjoining superficial muscle cells of the media. This organization gives the impression that the nerves penetrate the outer third of the media (cf. above). Here too the individual varicosities

frequently approach each other, coming as close as 200-250 Å membrane to membrane distance. As within the heart and iris, no synaptic membrane specializations similar to those found in the C.N.S. could be found, but in places where axon varicosities come in close apposition to each other, a slight thickening of adjoining membranes could occasionally be seen. The cleft between two adjacent varicosities or between a varicosity and a smooth muscle cell was always filled with a moderately electron-dense material, with an appearance suggesting that it could be comprised of the fused basement membranes of the including cells (cf. Thaemert 1963).

In middle cerebral arteries taken from normal, untreated cats, tyramine causes a contraction (paper 9). In arteries from cats that had been previously reserpinized (5 mg/kg intraperitoneally, 24 hours before sacrifice) or sympathectomized by removal of the superior cervical ganglion one week previously, no response was obtained until the concentration of tyramine in the bath was about 10^{-4}M, and the intensity of the concentration was then markedly reduced.

Although tyramine in low doses was without effect on reserpinized arteries, a normal contractile response could be elicited with the addition of NA (5×10^{-5}M) to the bath, in agreement with previous findings (Nielsen and Owman 1970). After 15 min., the noradrenaline was washed out from the bath, and it was afterwards possible to record a contraction with tyramine (10^{-5}M) on the previously reserpinized vessel. The contraction was of the same magnitude as in preparations from untreated animals. If cocaine (10^{-4}M) was added before the addition of NA (5×10^{-5}M) to preparations from reserpinized animals, it was impossible to restore the contractile effect of tyramine (10^{-5}M).

DISCUSSION

Heart, light microscopy

Classical investigations using silver and methylene blue staining techniques have demonstrated a rich network of nerve fibres in the heart (Stöhr 1957; Hirsch and Borghard-Erdle 1961; Hirsch 1963; Abraham 1964). However, the conclusions reached have been contradictory and at times difficult to relate to accepted physiological concepts. The adrenergic nerves to the heart have previously been investigated in some detail in mammals (Angelakos, Fuxe and Torchiana 1963; Dahlström, Fuxe, Mya-Tu and Zetterström 1965) and in some amphibians (Falck, Häggendal and Owman 1963; Angelakos, Glassmann, Millard and King 1965; Bannister and Mann 1966). In all essentials the adrenergic innervation patterns in mammals were confirmed in the present studies (paper 2 and 4). However, a previously unknown supply of a network of adrenergic fibres in the endocardium and the valves has also been demonstrated. Smooth muscles are well known in the endocardium and the heart valves, and it seems likely that they are innervated by the adrenergic fibres.

It is usually suggested that the existence of large amounts of AChE in a nerve is an indication of the nerve being cholinergic (cf. Koelle 1963). It has also been shown that adrenergic fibres in the rat and cat do not contain AChE in sufficient amounts to be demonstrated with the method used (Ehinger 1966 b, 1967) although small amounts of AChE may occur in non-cholinergic ganglion cells (Giacobini 1967). The choice of inhibitors, however, is of great importance for the specificity of the procedure. In cats, mipafox has been shown to inhibit ChE, and in the consentrations used, it is adequate for demonstrating AChE (Holmstedt 1957). Also, judged from the inhibition-curve of iso-OMPA, this substance seems well suitable as inhibitor of ChE (Diegenbach 1965). In rats and cats, selective denervations have settled the

specificity of the used method, either with mipafox or iso-OMPA as inhibitor (Ehinger 1966 b, 1967). There are also good reasons for assuming that NU-683 selectively demonstrates AChE in rabbits (Koelle 1955).

Sensory fibres contain small amounts of AChE (Koelle 1963). However, experiments on cat cornea have shown that the concentration is too low to be demonstrated with the short incubation times involved (cf. Ehinger 1967). It is therefore unlikely that sensory nerves have appeared to any great extent in the material.

The cholinergic innervation of the heart has been investigated in many species, but with incompletely characterized inhibitors. The general distribution of AChE-containing nerves in the myocardium agrees with earlier results (cf. Koelle 1963) and also with the general distribution of adrenergic nerves described above. The existence of a single-layered nerve net of AChE-containing fibres subendocardially and in the valves has also been established in this study. With conventional stainings, an endocardial and valvular nerve plexus has been shown, but it has been thought to be sensory in character (cf. Stöhr 1957; Abraham 1964; Miller and Kasahara 1964; Williams 1964). The functional importance of these adrenergic and AChE-containing fibres is unknown, although the innervation might indicate a more active role of the valves in cardiac haemodynamics than usually thought. It is also worth noting that the adrenergic and AChE-containing nerves form similar patterns. As the adrenergic nerves have been shown to contain an insufficient amount of AChE to be demonstrated by the used method (see above) it seems reasonable to suppose that the adrenergic and AChE -containing nerve fibres run closely together in the heart valves. This was further confirmed by electron microscopy (see below).

In the myocardium, the adrenergic and AChE-containing fibres appear in approximately similar proportions. Adrenergic as well as cholinergic nerve terminals contain ChE. If adrenergic and AChE-containing nerve terminals form different networks, sections stained to show ChE as well as AChE should contain more visible nerves (namely adrenergic plus cholinergic) than sections stained to show only AChE-containing fibres (cholinergic). That this was not the

case argues in favour of adrenergic and cholinergic fibres running close to each other not only in the valves, but also in the myocardium. The existence of such an organization was established with methylene blue technique and electron microscopy (Ehinger, Falck and Stenevi 1969; Sporrong unpubl. obs. and cf. below).

Most of the above-mentioned findings have later been verified in independent laboratories. Thus Jacobowitz, Cooper and Barner (1968) studied the normal and denervated cat heart with chemical and histochemical methods, including fluorescence and AChE techniques. They found an almost complete adrenergic denervation upon mediastinal neural ablation, which makes it highly probable that the adrenergic nerves to the heart of at least the cat are largely postganglionic. This agrees with our findings of only non-adrenergic (i.e. presumably cholinergic) intramural ganglion cells within the hearts of several species, and with the results of Nielsen, Owman and Rosengren (1968) and Nielsen and Owman (1969) using another denervation technique. However, with their denervation technique, Jacobowitz et al. (1967) also found a considerable reduction of cholinergic terminals in the left atrium and to a minor degree in the right atrium, leaving an intact cholinergic innervation pattern in the ventricles. This suggests that a part of the cholinergic innervation to the cat heart is postganglionic. Nielsen and Owman (1968), in their studies on the mechanism of ventricular fibrillation in hypothermia, noticed a marked difference in the number and distribution of adrenergic nerve terminals in the hearts of hibernators compared with non-hibernating animals. The SA and AV-nodes of the investigated hibernators contained an intense adrenergic innervation well comparable with that of non-hibernating animals. However, the heart ventricles of the hibernators contained only scattered adrenergic terminals mainly confined to vessels. Only a few, if any, terminals could be seen in the myocardium well away from vessels.

The adrenergic innervation patterns in the hearts of rats and guinea-pigs were investigated by Winkler (1969). The main distribution and localization of adrenergic nerve agree with the findings in the present investigation. However, in addition, it was claimed that

the density of the adrenergic terminals in the heart was greater in the guinea-pig than in the rat.

Chemical determinations of NA in cat hearts disclosed a higher content of NA in the ventricles than in the atria (Jacobowitz et al. 1967; Nielsen and Owman 1968). In the fluorescence microscope, our findings have not agreed with these chemical results. Instead, we found a denser adrenergic nerve plexus in the atria than in the ventricles. A possible explanation might be a higher NA content in the adrenergic terminals of the ventricles than in the atrial adrenergic terminals, or a tendency of the eye to perceive several terminals lying close together as one.

The adrenergic nerve plexus of the heart valves has also been described with essentially the same results as described above (Lipp and Rhodin 1968). With the aid of methylene blue combined with fluorescence (cf. material and methods, and below) Ehinger, Falck and Stenevi (1969) confirmed that adrenergic and cholinergic terminals ran distinctly separated, but close to each other, forming similar patterns in the autonomic ground plexus in the heart valves.

In the microspectrofluorometer, it is possible to distinguish NA from DA by their different behaviour upon treatment with HCl: the excitation peak of the dopamine fluorophore shifts to 370 nm and is not affected by excessive HCl-treatment, whereas the noradrenaline fluorophore only transiently exhibits an excitation peak at this wavelength (Björklund, Ehinger and Falck 1968). DA can be detected in the presence of NA even if its concentration is only 10 per cent of the NA concentration (Baumgarten, Braak and Falck 1968). Sometimes, a small peak at 370 nm was seen in adrenergic neurons of the heart. This could possibly signify the presence of small amounts of DA in the nerve fibres. However, since the nerve fibres undoubtedly contain high concentrations of NA, this 370 nm peak could also represent incompletely converted NA. In any event, the nerves in the SA-node, in the myocardium proper, and around vessels showed no differences. It is thus ques-

tionable whether special dopaminergic nerves occur in the sino-atrial node as proposed by Angelakos et al. (1963). Instead, it seems plausible that most of the dopamine found in the atria is situated in the small intensive fluorescent cells which have been shown to contain dopamine ~~found in the atria is situated in the small intensive fluorescent cells which have been shown to contain dopamine~~ (Björklund, Cegrell, Falck, Ritzén and Rosengren 1970). These cells have also been described by Jacobowitz (1967), who thought that they might function as some sort of intermediary link between nerve cell bodies in the cholinergic ganglia in around the heart. Since they have been shown to occur well away from ganglia and in many other places in and around the heart and in other organs as well (Björklund et al. 1970) it seems difficult to impute to them such a specialized function, particularly as the main amine they contain seems to be DA instead of NA.

Heart, electron microscopy

Earlier electron microscopic studies of the relations between nerve terminals and myocardial cells have produced contradictory results. Thus no close contacts of possibly synaptic character were found within the hearts of dogs (Napolitano, Cooper, William and Hanton 1964) or rats and rabbits (Hadek and Tolso 1967). However, close contacts between nerve terminals and heart muscle cells are described by Bozner, Barta and Mrena (1966) although they do not state whether these contacts are of synaptic character. More elaborate information on possible synaptic connexions is given by Thaemert (1966) in the heart of frog (Rana pipiens), by Novi (1968) in the rat papillary muscle, and by Thaemert (1969) in certain parts of the mouse heart. A rich supply of nerves has also been disclosed in the conduction system of the heart on the electron microscopical level (Trautwein and Uchizono 1963; Maekawa, Nohara, Kawamura and Hayashi 1967; Thaemert and Emmet 1968).

Axo-axonal contacts of the type described in the pre-

sent investigation can be found in the illustrations of previous papers, but usually without any comments (Bozner et al. 1966, Hadek et al. 1967) or only a short comment in passing (Thaemert 1966). Conceivably, the authors have refrained from comments because it was impossible to know the proper type of axons involved in the contacts, which then simply could represent the situation close to a branching of the terminal axon. In the present investigation, this difficulty has been overcome by the use of two different methods.

By the use of $KMnO_4$ as fixation medium of tissues from untreated animals, most or all synaptic 400-500 Å vesicles in peripheral adrenergic nerve terminals achieve a dense core, whereas the synaptic vesicles in non-adrenergic nerve terminals remain empty (Richardson 1966; Hökfelt 1967; Hökfelt et al. 1968). The specificity has been verified by pretreatment with reserpine or sympathectomy on the rat iris (Hökfelt 1967).

It has recently been shown that, after the injection of 5 hydroxydopamine, the normal transmitter in adrenergic nerves is replaced by this substance, ehich thus acts as a falce transmitter (Tranzer and Thoenen 1967). In the electron microscope, this can be noted after glutaraldehyde and osmium fixation by the appearance of highly electron-dense cores in the synaptic vesicles of adrenergic nerve terminals, whereas the synaptic vesicles in non-adrenergic terminals remain empty. The specificity of this has been ascertained by selective sympathetic denervations on the albino rat iris (paper 6). However, in contrast to the $KMnO_4$-fixed tissues where most or all synaptic vesicles obtain a dense core, a certain amount of the synaptic vesicles are usually empty in adrenergic nerves from 5-hydroxydopamine-treated animals. This can perhaps be explained by the recent finding of Ehinger and Sporrong (unpubl.) of a gradual substitution of NA by 5-hydroxydopamine in the adrenergic nerve terminals, and that adrenergic nerves in different organs have a different degree of sensitivity to 5-hydroxydopamine.

In the present investigation (paper 6,8), the innervation patterns from earlier light microscopical findings (paper 2,4; Ehinger, Falck and Stenevi 1969) were confirmed. The intense nerve supply to the SA node seen with light microscopy in most species, corresponds well to the present demonstration of the ample supply of preterminals and terminals in and around the rabbit SA node (paper 8). Nerve terminals partly or fully devoid of their Schwann cell sheathing and containing dense-cored or empty vesicles are frequently seen coming in close apposition to each other and to the sinus node cells, every muscle cell being reached by at least one and probably several nerve terminals of both the dense-cored and empty vesicle types. These close contacts between individual varicosities or between varicosities and the myocardial cell are characterized by a membrane to membrane distance of 200-250 Å, an occasional slight thickening of one or both membranes included, and a moderately electron-dense material filling the space between adjacent membranes. Thus all the criteria proposed by Thaemert (1963) as signs of a possible peripheral synaptic contact are fullfilled.

The atrial as well as the ventricular myocardium contains considerably less nerve terminals than the SA and AV nodes. In addition to the above-mentioned axo-axonal contacts visible in all parts of the heart, close contacts between nerve terminals and heart muscle cells of the type described by Thaemert (1963, 1969) were found in all parts of the heart that were examined. They were thus found also in the apical part of the rat heart, although less frequently than elsewhere. However, Thaemert (1969) failed to demonstrate close contacts between nerve terminals and heart muscle cells in the apical part of the mouse heart.

The existence of a valvular and subendocardial plexus of nerve terminals seen with the light microscope (cf. above) was confirmed. These plexuses contained terminals of both the dense-cored and the empty vesicle types, often coming in close contact to each other, the distance between adjacent membranes being in the order of 200-250 Å.

Adrenergic innervation of vessels

The vessels in most mammalian tissues have a sheath of adrenergic fibres in a plexus around the media (Falck 1962, Norberg and Hamberger 1964, Fuxe and Sedvall 1965, Folkow, Fuxe and Sonnenschein 1966, Cech and Dolezel 1967, paper 2, Schenk and El Badawi 1968, and others). Studies with the electron microscope have also shown that vascular nerves are confined to the junction between the adventitia and the media in muscular arteries in a variety of organs (Pease and Paule 1960).

Only rarely are adrenergic fibres seen to enter the media in the muscular arteries, although some limb arteries of the dog, for instance, seem to have an increased number of adrenergic terminals in the media (Tsunekawa, Mohri, Ikeda, Ohgushi and Fujiwara 1967, Gerova, Gero and Dolezel 1967). In large elastic vessels such as the pulmonary artery, adrenergic fibres can sometimes be observed between the elastic lamellae of the outer third of the media (paper 2, Bevan and Verity 1967). This may represent an innervation of the intrinsic vessel muscles, or the vasa vasorum, or both. Veins have a considerably less well-developed plexus of adrenergic terminals, but whole mounts of, for instance, the caval or renal veins (paper 2) reveal a significant number of terminals that form a loose-meshed plexus.

In vessels where much connective tissue separates the muscle fibres into separate bundles, adrenergic terminals can be seen throughout the media (paper 2, Loizon and Tindall 1968). Apparently, in most muscular vessels, excitation can spread inwards from the superficial cells without nerves, but when isolating connective tissue is present, a more elaborate nerve plexus seems to be necessary.

In skeletal vessels, capillaries are not reached by adrenergic fibres (paper 2).

In structures with many capillaries and many adrenergic terminals, it could not readily be distinguished in the light microscope whether the adrenergic terminals reach the capillaries or only

pass by, even though the latter alternative seemed most likely. In some organs, however, adrenergic terminals have been observed in a pattern suggesting an innervation of certain specialized capillaries (Ehinger 1966 c, Alm, Ehinger and Falck 1967, Cegrell 1968, Lever, Spriggs and Graham 1968).

In cerebral vessels, adrenergic terminals are numerous around the vessels forming the cricle of Willis, especially the anterior and middle cerebral arteries in rats and cats (Carlsson, Falck and Hillarp 1962, Nielsen and Owman 1967, Donáth 1967). A similar acetylcholinesterase-containing nerve net in the main pial arteries was shown by Lawrentieva, Mchedlishvili and Plechkova (1968). Hagen and Wittkowski (1969) have gi en an excellent ultrastructural description of the pial vascular nerves, but did not distingiush between adrenergic and non-adrenergic terminals. The present observations (paper 7) agree with those of Hagen and Wittkowski. In addition, both adrenergic and cholinergic fibres have been identified. These fibres often come in close apposition to each other, at times with faint membrane specializations, similar to that observed in the heart and iris (see below). Although often running denuded of their Schwann cell sheath, the axons never come closer to the vessel muscles than about 1000 Å. Strong contractions can be induced in feline pial vessels by a variety of biogenic amines, including noradrenaline (Nielsen and Owman 1970). Tyramine in moderate doses also induces strong contractions (paper 9) that can be blocked by reserpine or sympathectomy. Since tyramine acts by liberating the endogenous nerve noradrenaline, it is reasonable to presume that the adrenergic nerve plexus around the pial arteries contributes to a true innervation apparatus, and that it can produce a contraction of the vascular smooth muscle through a local release of the noradrenaline transmitter. It also follows that the noradrenaline can act over distances of at least about 1000 Å. Similar conclusions have been drawn from experiments with the pulmonary artery (Verity and Bevan 1966; Verity, Bevan and Ostrom 1966; Bevan and Verity 1966) or certain vessels in the pancreas (Lever and Esterhuizen 1961) and lung

(Fillenz 1969).

Relations between peripheral adrenergic and cholinergic axons

Previous light microscopical investigations with cholinesterase stainings showed a network of adrenergic and acetylcholinesterase-containing fibres in the rat iris, with the two fibre types presumably running closely intertwined (Ehinger and Falck 1965, 1966). Simultaneously, methylene blue staining showed (paper 1) that several axons usually ran together in the mouse iris, whereas fluorescence microscopy demonstrated that the adrenergic axons usually ran single. If the methylene blue staining revealed both adrenergic and cholinergic fibres, it would seem that an adrenergic axon is usually accompanied by a cholinergic axon. Subsequent denervations (paper 3) have shown that methylene blue stains cholinergic fibres preferentially. However, in the mouse iris study, staining was not judged acceptable if the iris vessels, which have a purely adrenergic innervation (Ehinger and Falck 1966), did not show a well-stained nerve plexus, and it is thus unlikely that the adrenergic fibres would have remained unstained in this case.

Direct evidence that cholinergic and adrenergic terminals run together was obtained with methylene-blue staining combined with fluorescence microscopy after formaldehyde treatment. It was noticed that under certain conditions (paper 3) two groups of fibres appeared in whole mounts of the rat iris: one stained with methylene blue, and one displaying the fluorescence specific for catecholamines. Along the vessels, only fluorescent adrenergic fibres appeared. After selective parasympathetic denervation, most fibres stained with methylene blue disappeared, whereas the adrenergic fibres showed no appreciable change. Thus, preferentially, cholinergic fibres stain with methylene blue. The adrenergic fibres can also be stained with methylene blue, but they then lose their fluorescence. The fluorescence loss occurs well before the fibres stain.

The observation that methylene blue stains cholinergic fibres preferentially has been confirmed by electron microscopy (Richardson 1968). After such staining and potassium permanganate fixation, cho-

linergic axons become less electron translucent in parts near the membrane, suggesting that this is the site of the staining.

The reason methylene blue preferentially stains cholinergic fibres is unknown. The staining is best performed at pH 6 (Richardson 1969), but is otherwise remarkably resistant to drugs such as ouabain, KCN, cocaine, hexamethonium, acetylcholine or eserine, to cold (+4°C), to glucose deficit, to variations in the K^{+}, Na^{+}, Cl^{-}, Mg^{++}, and Ca^{++} ions of the staining bath (Ehinger, Sporrong and Stenevi, unpublished) and to osmotic variations (Richardson 1969). Some improvement in the staining is seen if stained tissue pieces are exposed to increased O_2 partial pressure, but oxygen deprivation during the actual staining seems to be of little importance (Ehinger, Sporrong and Stenevi, unpublished). Staining is satisfying in thin tissue sheats such as the iris, heart valves, and mesentery, but only near the surface of perenchymatous organs such as the heart and salivary glands. From the experiments with drugs, it is clear that the staining does not depend on any energy-requiring mechanisms such as a membrane "pump". The staining differences between adrenergic and cholinergic fibres are perhaps due to differences in passive membrane permeability, in the ability to keep methylene blue in the oxidized stained form, or in the chemical binding of the stain.

In addition to the results from the iris, concomitant adrenergic and cholinesterase-containing fibres were demonstrated in heart valves with the light microscope (paper 4, see above). Subsequent stainings with methylene blue (Ehinger, Falck and Stenevi 1969) as well as electron microscopical studies (paper 6, see above) have confirmed the observations. In the myocardium, AChE studies again suggested that adrenergic and cholinergic fibres run tightly intertwined (paper 4, see above).

It was subsequently confirmed by electron microscopy that adrenergic and cholinergic terminals not only run together in the same Schwann cell sheath in the myocardium, but also in cardiac valves, in the iris (paper 6), and in certain pial vessels (paper 7).

Moreover, they often come in close proximity to each other, membrane to membrane, with only 200-250 Å interspace. Similar contacts between peripheral adrenergic and cholinergic nerves have been observed by Thoenen et al. (1966) in the vas deferens and iris (Ochi, Konishi, Yoshikawa and Suno 1969). Sometimes, faint thickenings of the membranes were observed at such places of contact, and also a weak increase in the electron opacity of the intermembraneous substance. These structure specailizations are not of the magnitude seen in the so-called synaptic structures in the brain. However, as has been pointed out (paper 6), it is not known to what extent such special structures are required in a synapse. They do not usually occur at synapses between peripheral autonomic nerves and their effector cells. Thus the place where an axon containing mitochondria and synaptic vesicles approaches another axon with only 200-250 Å interspace may be regarded as a presumptive axo-axonal synapse. Obviously, the definite support for such a synapse must rely on pharmacological and physiological experiments. This is further discussed below.

Taken together, the studies with the light and electron microscope demonstrate that adrenergic and cholinergic axons to a considerable extent run closely together in the iris, heart, vas deferens, and certain vessels. Very often contacts of possibly synaptic character occur between adrenergic and cholinergic axons.

The cholinergic link hypothesis

It is only in recent years that considerable and important revisions have been made in the classical concepts of the principles of constructions of the peripheral vegetative nervous system. The previous rigorous division of it into a cholinergic, parasympathitic part has proved to be too schematic. As early as 1931, von Euler and Gaddum showed that acetylcholine was released from certain sympathetic nerve trunks, and subsequently acetylcholine-releasing fibres have been demonstrated in a great number of different sympathetic nerve trunks (splenic nerve, sympathetic nerves to the heart, nictitating

membrane, intestine skin vessels, hindleg vessels, and others; for ref., see Burn and Rand 1965 and Ferry 1966). Further, both cholinergic and adrenergic terminal networks have been demonstrated in a number of organs where there was previously some doubt, such as in the pupillary muscles (e.g. Laties and Jacobowitz 1964, 1966, Ehinger and Falck 1965, 1966, Ehinger 1964, 1966 a and b, 1967, Hökfelt 1967, Richardson 1969, Roth and Richardson 1969, Ehinger, Falck and Sporrong 1970), the heart (Ehinger, Falck and Sporrong 1968, 1970, Jacobowitz, Cooper and Barner 1967), the nictitating membrane (Jacobowitz and Koelle 1965, Ehinger 1966 d), the vas deferens (Jacobowitz and Koelle 1965, Robinson 1969) or at certain vessels (Lever, Spriggs and Graham 1968, Lawrentieva, Mchedlishvili and Plechkova 1968, Fillenz 1969, Nielsen, Owman and Sporrong 1970, Nelson and Rennels 1970, Waterson, Hume and de la Lande 1970). This agrees well with the observations of acetylcholine-containing axons in most sympathetic nerve trunks (see above). Clearly, interpretations of physiological or pharmacological experiments on the function of the vegetative nervous system must show that the rigorous and schematic separation of it into two separate entities is not tenable; in reality, it is usually composed of a complex system of both adrenergic and cholinergic fibres.

Further complications arise because the innervation of the vas deferens and the intestine has been found to depart significantly from the simple scheme previously adopted. In neither case can a purely postganglionic adrenergic effect be obtained by stimulating nerves leading to the organ. The adrenergic terminals of the vas defernes arise in peripheral ganglia close to the organ (Owman and Sjöstrand 1965) and the situation is much the same in all parts of the internal male and female genital organs (Sjöstrand 1965, Owman, Rosengren and Sjöberg 1966). In the intestine, by far most of the adrenergic terminals end in the myenteric plexus, and only few, if any, reach the muscles directly (Norberg 1964, Jacobowitz 1965,

Read and Burnstock 1968, 1969, Gabella and Costa 1969).

Clearly, most or all experimental preparations with sympathetic nerves from the abdominal viscera are in reality nerve--ganglion-muscle preparations, although often erroneously regarded as simple nerve-muscle preparations. There are further complications because the transmitter substance of the inhibitory axons originating in the myenteric plexus is unknown (see Burnstock et al. 1964, Furness 1969). In a large number of cases, these complications invalidate, or at least cast doubt upon, the conclusions drawn from experiments with abdominal nerve-viscera preparations.

The observations fundamental to the hypothesis of Burn and Rand, of a cholinergic link in the release of noradrenaline from adrenergic terminals, were that acetylcholine had certain sympathetic effects and that acetylcholine could be released from sympathetic nerve trunks. It was originally suggested that the transmitter, noradrenaline, might be situated outside the nerve terminals, but the fluorescence histochemical reaction of Falck and Hillarp (Falck 1962, Falck, Hillarp, Thieme and Torp 1962) proved that the nerve terminals are the main site of the transmitter.

The hypothesis has stimulated several types of investigations. It has been established beyond doubt that acetylcholine can be released from a great number of sympathetic nerve trunks (cf. above). It has further been shown that acetylcholine can release catecholamines from adrenergic terminals (Thoenen et al. 1966; Cabrera, Torrance and Viveros 1966; Haeusler et al. 1968;Burn and Rand 1965; Ferry 1966). The adrenergic terminals thus appear to possess receptors that can be blocked by hexamethonium and presumably also d-tubocurarine (Ferry 1966, Dempsey and Cooper 1969). Acetylcholine applied exogenously to the terminals is also able to set up antidromic volleys in sympathetic cardiac nerves (Haeusler et al. 1968). Moreover, there is ample evidence that substances with an anti-cholinesterase effect can enhance the response to stimulation (see Burn 1968). There is no disagreement on

these points, and except for the hexamethonium and d-tubocurarine effect, they are clearly compatible with a cholinergic mechanism in the release of noradrenaline from adrenergic terminals.

The blocking effect of hexamethonium and d-tubocurarine on the noradrenaline release by acetylcholine is not readily explained by the Burn and Rand hypothesis. Burn suggests that bretylium, an anti-adrenergic drug which can also inhibit the neuromuscular junction, acts by inhibiting the postulated cholinergic link in the adrenergic terminals. Hexamethonium should then also inhibit the adrenergic terminals, but this does not happen. Burn and Rand (1965) proposed that the discrepancy might be explained by different penetration abilities of the two drugs, bretylium and hexamethonium. The explanation further requires the cholinergic mechanism to be entirely intra-axonal. An explanation is then needed of why acetylcholine is released in detectable amounts from the axon when it is stimulated. A summary of these points can be found in Burn and Rand (1965) or Burn (1968 b).

There have been many results whose interpretation opposes the Burn and Rand hypothesis. In part, these studies have been performed with hemicholinium in favour of the hypothesis. Ferry (1966) and Thoenen, Tranzer, Huerlimann and Haefely (1966) have given summaries. Of the evidence opposed to the hypothesis, it can particularly be noted that, in a preparation shown morphologically to be purely adrenergically innervated, anticholinesterases had no potentiating effect on sympathetic stimulation (Thoenen et al. 1966).

Adrenergic neuroeffector transmission can be blocked by many drugs that also block cholinergic transmission; this has been advanced as evidence for the cholinergic link hypothesis (see Ferry 1966). A number of these substances have been tested on abdominal viscera preparations, which usually contain a ganglionic synapse (see above) and the interpretation of the results are already complicated from this aspect. Moreover, to quote Ferry 1966, p. 433: "the fact that a substance known to block cholinergic trans-

mission also blocks adrenergic transmission need not necessarily imply participation of acetylcholine in the release of the sympathetic transmitter. The evidence merely indicates the possibility of a common mode of action of these substances in cholinergic and adrenergic fibres, and this may well be depression of the mechanism of impulse conduction in nerve terminals."

Koelle (1962) offered a somewhat different hypothesis concerning the function of the acetylcholine. Since the enzyme acetylcholinesterase is believed to have the rapid destruction of acetylcholine as its primary function, it should be localized to the site of action of the acetylcholine, removing "used" transmitter substance. It has been observed that in certain neurons, e.g., in the stellate ganglion, there is a presynaptic pool of acetylcholinesterase. It was then proposed that the nerve impulse first releases a minor amount of acetylcholine upon its arrival at the terminal. The released acetylcholine in turn reacts on the terminal, releasing more acetylcholine sufficient to influence the postsynaptic membrane. The hypothesis was mainly evolved concerning cholinergic terminals, but it was suggested that acetylcholine could also act in a similar way in adrenergic terminals, the only difference being that the initial release of acetylcholine triggers a release of noradrenaline. The acetylcholinesterase present in adrenergic terminals is small, however (cf. Ehinger and Falck 1966; Ehinger 1967), and considerably smaller than in cholinergic terminals. Acetylcholinesterases are also well known to occur at sites definitely devoid of nervous mechanisms, such as the myotendineal junction or in erythrocytes (see Koelle 1963). The demonstration of acetylcholinesterases also largely depends on specific inhibitions of non-specific esterases, and it must be noted that it is difficult to ensure complete such inhibition. The theory does not explain why hexamethonium is able to block the acetylcholine-induced release of noradrenaline from adrenergic terminals without blocking the response to electrical sympathetic stimulation. Discrepancies in the density of adrenergic and acetylcholinesterase-containing terminals also forced Jacobowitz and Koelle (1965)

to suppose that the presumed cholinergic mechanism in adrenergic terminals might be of different importance in different organs and species.

The hypothesis of a cholinergic link in the release of noradrenaline in adrenergic terminals was evolved under the then prevailing concept that the terminal network was constructed with separated adrenergic and cholinergic terminals. As pointed out above, this concept has been disproved; instead, the two types often lie together with contacts that could represent axo-axonal synapses (paper 6). Clearly, such an arrangement offers an alternative explanation to most of the results discussed above. The cholinergic fibres possibly release acetylcholine that acts on the adrenergic fibres. Based on experiments with HC-3, Leaders (1963) advanced a similar proposal. Thoenen et al. (1966) studied the cat spleen pharmacologically, and the cat spleen, iris, and vas deferens in the electron microscope, and also suggested that cholinergic fibres could influence the adrenergic fibres. The prerequisities necessary for such an interaction are at hand: several organs have presynaptic cholinergic nerve structures with mitochondria and synaptic vesicles coming as close to adrenergic axons as 200-250 Å (paper 6, and 8), and on the adrenergic terminals, there are receptors sensitive to acetylcholine (see above). Hexamethonium, d-tubocurarine, or any other substance might block these receptors without necessarily blocking the transmitter release by the nerve impulse. Also, eserine or any other anticholinesterase drug increases the effect of acetylcholine released from the cholinergic fibres, but the effect should be absent when cholinergic fibres are absent, as seems to be the case in the cat spleen capsule (Thoenen et al. 1966). Obviously, the finding that cholinergic terminals are widespread in most parts of the vegetative nervous system readily explains the original observation on which the hypothesis of Burn and Rand was developed, i.e. the release of acetylcholine from sympathetic nerve trunks.

Of the two alternatives, interaction between peri-

pheral nerve termiaals or cholinergic link in adrenergic terminals, the former seems simpler and thus more attractive. It must be observed, however, that this does not exclude the possibility of a cholinergic link. Direct evidence for it is lacking, however, and there are no experiments aimed at distinguishing between the two alternatives. A few attempts have been made to find choline acetylase (Nordenfeldt 1965, Ehinger et al. 1966) in adrenergic terminals. Both failed, but in both cases non-nervous enzyme activity might have masked a minute amount of choline acetylase. An attempt has therefore been made to measure the acetylcholine content of adrenergic terminals (paper 5). The cat iris can be selectively denervated parasympathetically (Ehinger 1967) leaving practically only adrenergic terminals. No acetylcholine was found in such irides, and it could be inferred that the content must then be below 6 per cent of normal (see above). The choline acetylase and acetylcholine determinations this show that if there is a cholinergic mechanism in adrenergic terminals, the amount of transmitter or its synthesizing enzyme is much less than in other nervous transmitter mechanisms. It also appears that the cell bodies of sympathetic neurons lack choline acetylase (Feldberg 1943, Banister and Scrase 1950, Hebb and Waites 1956). Moreover, Giacobini (1967) has shown that sympathetic ganglia contain both choline acetylase and AChE, and that preganglionic denervation results in a loss of choline acetylase activity which can be as much as 97 per cent of the normal value. Choline-acetylase is thus localized mostly preganglionically and most probably restricted to the preganglionic cholinergic fibres.

As the above discussion shows there is much evidence compatible with an influence from cholinergic terminals on the adrenergic terminals, at least under the conditions of the various experiments. From a strict morphological standpoint, the reverse influence is equally valid. However, there are far less pharmacological or physiological results in support of this. Leaders (1963)

found a cardio-inhibition following the classical cardiostimulatory response to sympathetic nerve stimulation. This inhibotory response was absent when the cholinergic fibres had been inhibited by HC-3, which indicates that adrenergic fibres could stimulate the cholinergic fibres to release ACh. Ehinger, Falck and Persson (1967 and 1968) noted a potentiation by atropine and hexamethonium on the response of the cat iris dilator to noradrenaline. This potentiation was absent after parasympathectomy, which suggests that the cholinergic terminals could be influenced by noradrenaline. Beani, Bianchi and Crema (1969) noted an inhibition by catecholamines on the release of ACh from guinea-pig colon; they postulated that this was due to a direct effect on the cholinergic nerve terminals. Actually, the inhibitory actions of catecholamines on the release of intestinal ACh or on ACh from the superior cervical ganglion are well established (see Beani _et al_. for references). Indirect evidence also exists that catecholamines and tyramine can release ACh into the vascular bed (Chandra, Dhawan and Gupta 1965; Kulkarni, Dadkar, and Gaitonde, 1965; Mc Carty and Chenoveth, 1966).

From the pharmacological and morphological results discussed above, it seems reasonable to presume that in the peripheral vegetative terminal nerve plexus both adrenergic and cholinergic terminals exert a mutual influence by means of axo-axonal contacts of possibly synaptic character. Such axo-axonal contacts do not exclude the possibility that each nerve terminal type also directly influences the effector cell.

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